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245 - A Highly Sensitive Glucose Electrode Using Glucose Oxidase Collagen Film *

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Summary

Industrially reconstituted collagen films have shown excellent properties for β–*D*–glucose oxidase coupling. Associated with a platinum anode for amperometric detection of hydrogen peroxide, these enzymatic films form a very simple and easy to handle glucose electrode ; this device presents a very high sensitivity (*ca.* 10^{-8} *M*) giving responses proportional to glucose concentration over 5 orders of magnitude.

Introduction

The association of an enzyme in a soluble form with an electrochemical sensor was first reported by Clark and Lyons, in 1962.[1] Since this date, the obtainment of carrier–bound enzymes has permitted the design of enzyme electrodes. In most cases, enzymes are trapped in gels surrounding the sensor[2] leading to systems which are difficult to handle.

In the present work, an enzymatic membrane was prepared from reconstituted calf skin collagen after acyl–azide activation and a coupling process giving a surface binding of enzymes of different classes.[3-5] A glucose electrode using β–*D*–glucose oxidase (GOD) membranes was developed, using an amperometric method with a platinum electrode to detect hydrogen peroxide, which is a product of the enzymatic oxidation of glucose according to the reaction :

$$\beta\text{-}D\text{-glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

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The potential was fixed at +650 mV *vs* Ag|AgCl, KCl 0.1 *M* and the anodic current was recorded. A stationary response was then obtained allowing the measurement of very low glucose concentrations. In a more sophisticated device, a second electrode involving a non–enzymatic membrane was used to compensate for the detection of other electroactive molecules and to enhance the selectivity of the glucose electrode.[6,7]

Experimental

Instrumentation

The glucose electrode consisted of a modified gas electrode in which the pH detector was replaced by a platinum disk and the usual teflon film by a collagen membrane. In a differential device, electrode 1 was mounted with a β–*D*–glucose oxidase collagen membrane and electrode 2 with a non–enzymatic one.

Electronics were made by Solea Tacussel : current outputs of both working electrodes were first subtracted (Deltapol) and then twice differenciated (Derivol) with a time–base of one second (GSTP) ; thus different current *vs* time curves were avaliable and usually recorded after a glucose pulse (Solea Tacussel EPL 2 with TV 11 GD plug–in unit, and three traces Linear 395 recorders).

Unless otherwise mentioned, the temperature of the solutions were carefully thermostated to 30.0±0.1 °C (Colora cryothermostat WK 5 DS).

Solutions and reagents

Insoluble films of highly polymerized reconstituted collagen (20 cm wide) were a gift of the Centre Technique du Cuir, Lyon (France) ; their thickness is about 0.1 mm in a dry state and 0.3–0.5 mm when swollen. They do not need to be tanned and can be stored several years without damage.[3-5]

Unless otherwise mentioned, all chemicals were reagent grade. The stock solutions of 0.1 *M* glucose were allowed to mutarotate at room temperature at least 3 hours before using and were stored at 4 °C. Both electrodes were filled with and dipped into 0.2 *M* acetate buffer, 0.1 *M* KCl solutions, pH 5.6.

Glucose oxidase binding on collagen membranes

The mild general acyl azide procedure for collagen membranes activation was used[3-4] followed by the enzyme coupling.

Carboxyls were first esterified by immersion of crude membranes in a methanol/0.2 *M* hydrochloric acid solution for at least 72 hours, then treated overnight by 1 % hydrazine and soaked at 4 °C for 3 minutes in 0.5 *M* $NaNO_2$ — 0.3 *M* HCl mixture just before coupling. Thorough washings were performed between each step and at the end of the activation process avoiding contact between reagents and enzyme solutions.

Activated films of 2×1.5 cm^2 were dipped (2 hours at 4 °C) into 1.5 cm^3 of borate buffer at pH 9 containing 2.5 mg β–*D*–glucose oxydase (GOD, E.C. 1.1.3.4, Boehringer lyophilizate, grade I). Excess of soluble glucose oxidase was washed away for about 100 minutes in 1 *M* KCl and surface activity was in the range 30–60 nmol. min^{-1} cm^{-2}. Enzyme collagen membranes were stored in 0.2 *M* acetate buffer, 0.1 *M* KCl (pH 5.6).

Procedure

Both electrodes were allowed to equilibrate in the buffer solution for 15 to 30 minutes after stepping the potential of the platinum disks to +650 mV *vs* Ag|AgCl, 0.1 *M* KCl. This potential corresponds to a diffusion–limited current for H_2O_2 oxidation. Calibrations were performed by successive microadditions (10 to 50 mm^3) of stock solutions of 10^{-6} to 10^{-1} *M* glucose to 10 to 20 cm^3 of buffer. The stationary response was the variation of the steady state values of I_1–I_2 when a sample of a glucose containing solution was added and the dynamic response was the height of the peak of the first derivative $d(I_1-I_2)/dt$. When successive additions were performed in the same solution, a current offset was used.

Results and discussion

When the enzyme electrode is immersed in a medium in which a pulse of glucose is added a steady state takes place after 2–3 minutes, as shown on Fig. 1, and the value of the anodic current reaches a plateau. The variation of intensity is directly dependent on the glucose concentration in the assay : this is the *stationary response*. On the other hand, the *dynamic response* is measured by the height of the peak obtained after 30–50 s by recording the first derivative of this current.

The lowest glucose concentration detected under these conditions is less than 10^{-8} *M* (Fig. 2). For values higher than 10^{-2} *M*, the responses become independent of the glucose concentration. The concentrations, which can be determined, range between *ca.* 10^{-8} and 10^{-2} *M* *i.e.* over 6 orders of magnitude and the linearity of the calibration curve is obtained between $(3–5) \times 10^{-8}$ and $(3–5) \times 10^{-3}$ *M* *i.e.* over 5 orders of magnitude (Fig. 2). 2.5.1

In a typical experiment, the calibration curves remained linear even after 40 hours operation at 30 °C and 250 days storage at 4 °C, allowing accurate repeated determinations for 160 micro–assays tested. However, a daily calibration was necessary, because of the slight decreasing of the calibration curve slope : *ca.* 20 % after 20 hours operation (cumulated time of measurements) and 20–50 % after 40 hours. The accuracy of glucose determinations during a set of experiments was tested by successive additions of the same aliquot of glucose (Fig. 1) ;

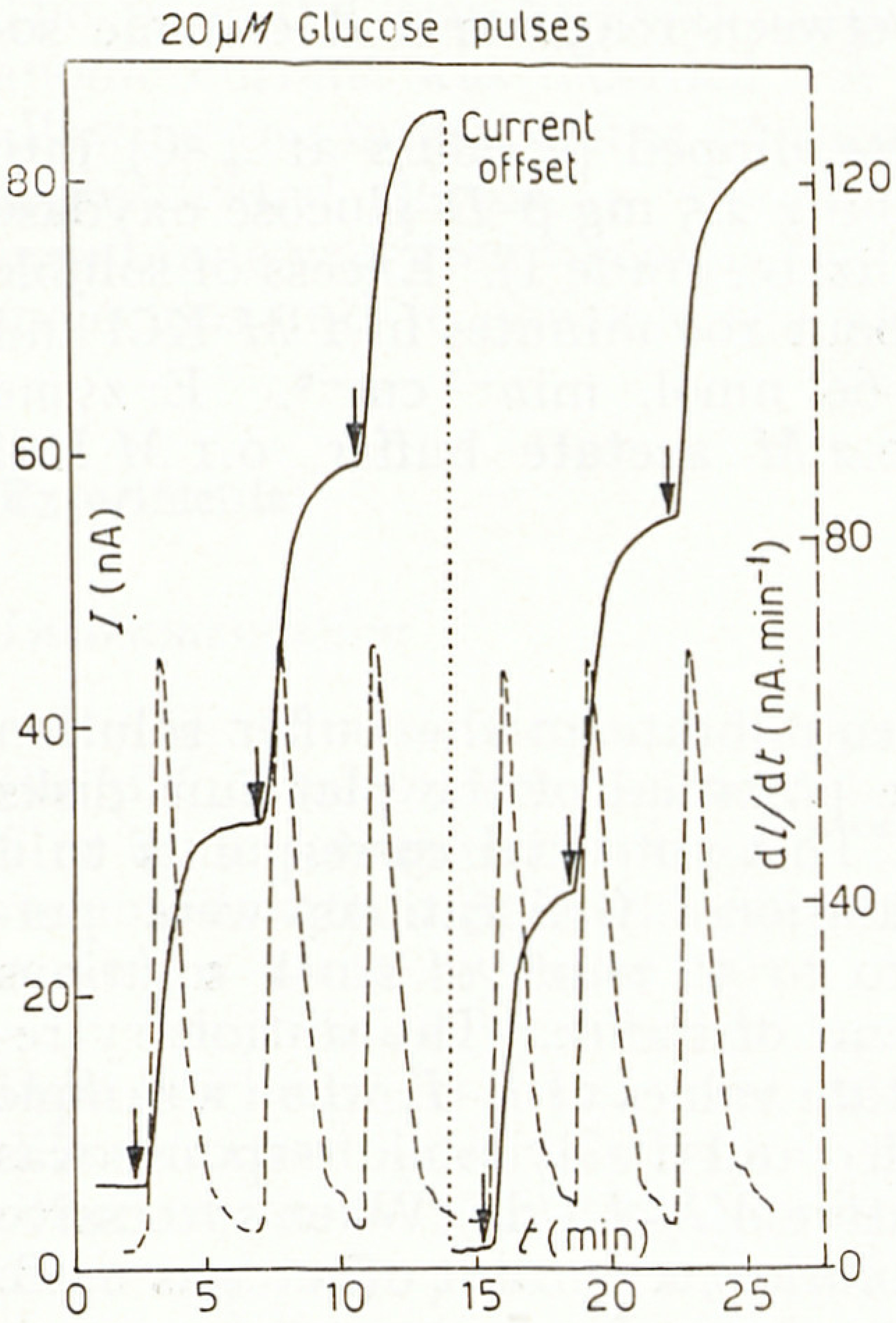

Fig. 1.
Electrode responses when successive 20 μM glucose pulses are added to the buffer solution. Addition of 40 mm³ (µl) aliquots of 10 mM glucose solution into 20 cm³ of 0.2 M acetate buffer, 0.1 M KCl (pH 5.6) solution. (———) direct current giving stationary responses, (— — —) first derivative of this current giving dynamic responses.

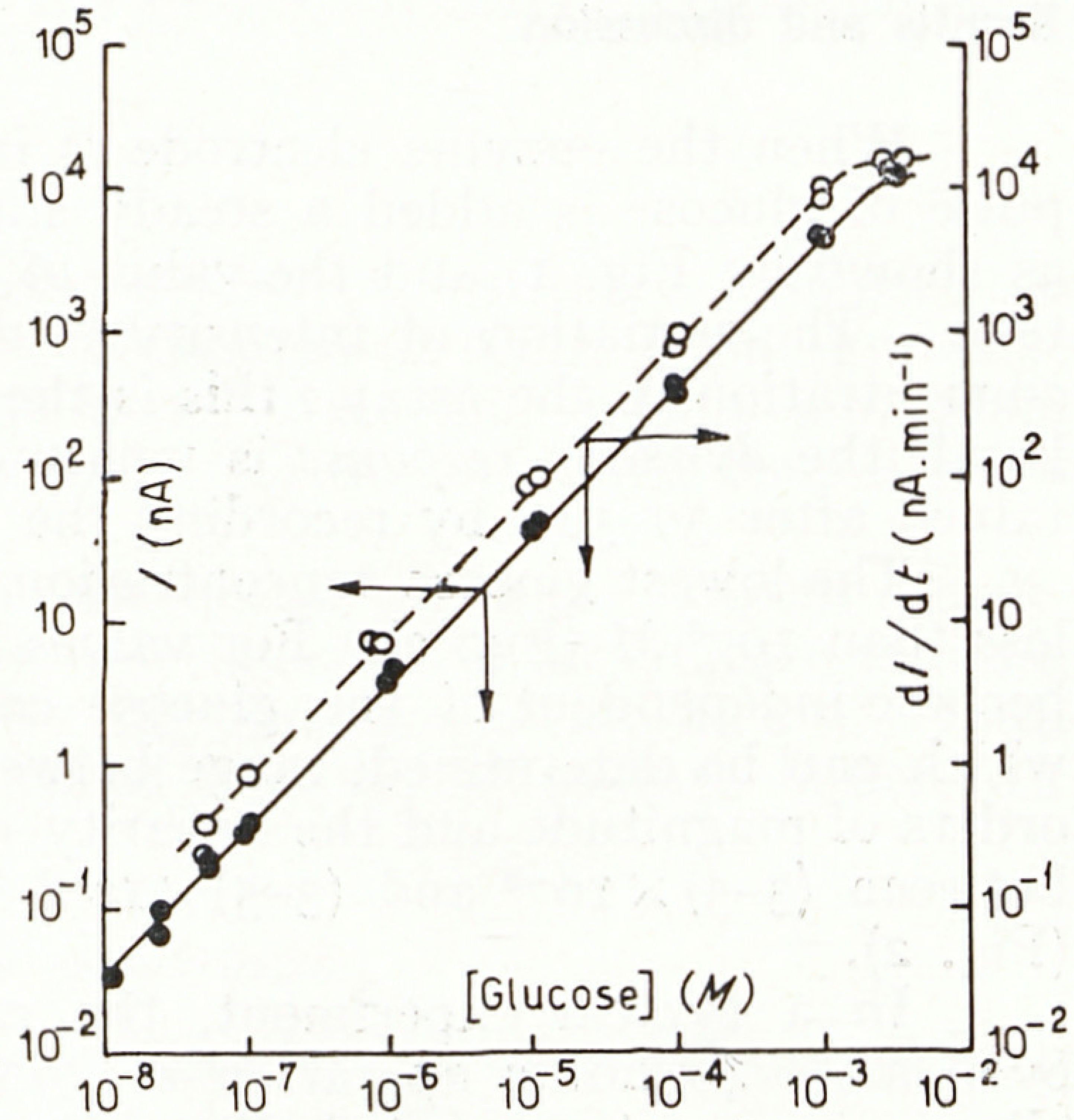

Fig. 2.
Calibration curves of the glucose electrode. (———) stationary response ; (— — —) dynamic response.

2.5.1

for 13 successive additiors of 10^{-5} M glucose, the standard deviation from the mean is usually lower than 2 %.

Glucose oxidase itself is very selective for β-D-glucose ; thus enzymatic electrode 1 presents a high selectivity for glucose compared with usual sugars : selectivity ratios are higher than 2 000 / 1 for fructose, lactose and sucrose. As other species may diffuse through collagen membranes and may be oxidized on platinum at +0.94 V (N.H.E.), the use of a compensating non enzymatic electrode 2 eliminates possible interferences of species such as ascorbate, urate, tyrosine or hydrogen peroxide; the selectivity ratio for hydrogen peroxide ranges between 80/1 and 250/1 depending upon experimental parameters (accuracy of the balance of both electrodes and GOD activity of the film).

The use of non-enzymatic electrode 2 is specifically of greatest interest for blood glycemia determinations, I_2 usually reaching 10–50 % of I_1–I_2. Fig. 3 presents the typical analysis of blood plasma samples after induced glycemia : in this case, a glucose calibration is necessary for each set of 3–4 blood additions. The dotted line, representing the second derivative of the current I_1–I_2, has been successfully used for monitoring a printing device of the peak of the first derivative, *i.e.* the dynamic response.[7]

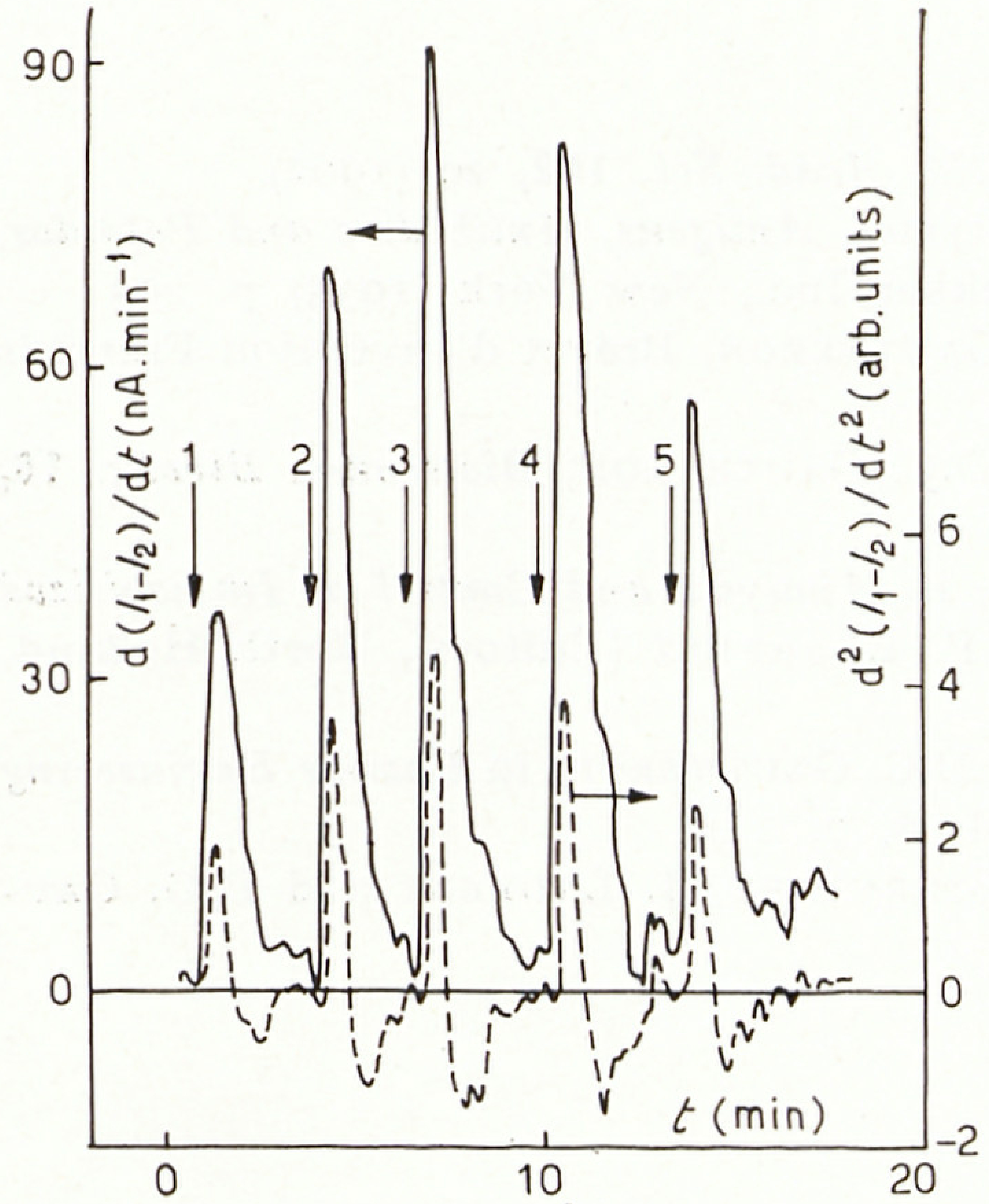

Fig. 3.
Induced glycemia. (———) first and (— — —) second derivative of the current after injection of 50 mm³ (μl) non deproteinized plasma samples. to 20 cm³ buffer (1) calibration of the electrode with a 10 μM glucose pulse, (2–5) glucose determination in plasma samples taken after 0 (2), 30 (3), 60 (4) and 120 minutes (5) after induced glycemia. Corresponding glucose content were respectively 1.1, 1.7, 1.4 and 1.0 g/l.

The glucose electrode may be used in a large temperature range, from 15 to 40 °C. As both responses are very sensitive to temperature (about 4–5 % / °C at 30 °C) it is necessary to carefully thermostate the solutions in contact with both electrodes.

Conclusions

Industrially reconstituted collagen films were found suitable for glucose oxidase immobilization ; the enzymatic activity was maintained and its stability enhanced. A membrane loading of 50 to 100 mU per membrane (1 cm diameter) was sufficient to obtain a very sensitive glucose electrode when associated with amperometric hydrogen peroxide detection. Stationary and dynamic responses of this glucose electrode was proportionnal to glucose concentration from $3-5\times10^{-8}$ to $3-5\times10^{-3}$ M. Furthermore, this sensor was used for whole blood samples analysis in induced glycemia.

Acknowledgements

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